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ORIGINAL RESEARCH

Emerging Mobile Colistin Resistance Gene *Mcr-1* and *Mcr-10* in *Enterobacteriaceae* Isolates From Urban Sewage in China

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Purpose: This study aimed to investigate the epidemiology and dissemination of *mcr*-positive *Enterobacteriaceae* in urban sewage in Yangzhou, China.

Methods: A total of 366 sewage samples were collected from the Yangzhou Wastewater Treatment Plant in Jiangsu Province. Colistin-resistant *Enterobacteriaceae* was identified through PCR targeting *mcr-1* to *mcr-10* genes. The isolates underwent antimicrobial susceptibility testing, and whole-genome sequencing was performed to analyze their genomic features. Additionally, conjugation experiments were conducted to assess the transferability of *mcr*-positive plasmids.

Results: Three *mcr*-positive *Enterobacteriaceae* isolates were identified, representing an isolation rate of 0.82%. These included one *mcr-1*-positive *Escherichia coli* (ST167) and two *mcr-10*-positive *Klebsiella pneumoniae* complex strains with novel sequence types ST6801 and ST6825. The *mcr-1* gene was located on an IncI2 plasmid (pYZ22WS208_3) and successfully transferred to recipient strains. In contrast, the *mcr-10* gene was carried on IncF plasmids (pYZ22WS067_1 and pYZ22WS223_1) but was not transferable in this study. Phylogenetic analysis revealed that the *mcr-1*-positive *E. coli* strain clustered within Clade II, alongside strains from various countries and sources. Phylogenomic analysis of *mcr-10*-positive isolates showed their sporadic distribution across 13 countries, with associations to diverse hosts and environments, indicating potential for widespread transmission.

Conclusion: This study demonstrates the presence of *mcr-1* and *mcr-10*-positive *Enterobacteriaceae* in wastewater, emphasizing the importance of wastewater surveillance for tracking antibiotic resistance. The horizontal transfer of *mcr-1* and potential spread of *mcr-10* across various hosts underscore the need for ongoing monitoring and preventive measures.

Plain Language Summary:

1. Colistin resistant *Enterobacteriaceae* isolates were identified in urban sewage.
2. The common genotypes revealed were *mcr-1* and *mcr-10*.
3. Urban sewage may serve as a reservoir for antibiotic resistance genes, presenting a significant risk to public health.

Keywords: *mcr*, *Enterobacteriaceae*, urban sewage, antibiotic resistance, whole genome sequencing

Introduction

The rise of multidrug-resistant (MDR) bacteria poses a significant threat to clinical care and global public health.[1–3] Among these, carbapenem-resistant *Enterobacteriaceae* has been categorized as critical priority pathogens by the World Health Organization (WHO) due to their limited treatment options.[4] The emergence of plasmid-mediated colistin resistance genes, such as *mcr-1*, has further exacerbated this issue by compromising colistin's effectiveness as a last-

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resort antibiotic.[5] The increased use and misuse of colistin in livestock farming has inevitably led to the emergence of colistin-resistance isolates.[6,7]

Mobile colistin resistance gene, *mcr-1*, first identified in *Escherichia coli* from a pig in China in 2015,[8] has since been reported globally across various bacterial species and hosts, including humans, animals, and the environmental sources.[9] The horizontal transfer of *mcr* genes via plasmids facilitates the rapid spread of colistin resistance.[10] To date, ten different variants of the *mcr* gene family (*mcr-1* to *mcr-10*) have been identified, reflecting the diversity of mechanisms that contribute to colistin resistance.[8,11–19]

The *Klebsiella pneumoniae* complex comprises species such as *K. pneumoniae, K. quasipneumoniae, K. variicola*, and *K. Africana*.[20] These Gram-negative opportunistic pathogens are commonly associated with infections in hospital settings[21–25] and is frequently implicated in the dissemination of *mcr* genes.[8,16,26–34]

Recent studies have highlighted the emergence of colistin-resistant strains in various environments, including wastewater treatment plants. For instance, Cherak et al identified MCR-1 producing Gram-negative bacteria in aquatic settings in Algeria,[35] while Snyman et al discovered *mcr-3* and *mcr-5* positive *Aeromonas spp*. in water sources in South Africa.[36] Additionally, Puljko et al isolated *mcr-4.3* positive *Klebsiella spp*. from treated wastewater in Croatia.[32] These findings underscore the role of wastewater plants as critical junctures for the dissemination of antibiotic resistance, integrating antibiotics excreted in urine and feces from both human and veterinary sources, and ultimately facilitating the transfer of resistance genes into the environment.[37–40] While colistin resistance in wastewater is a global concern, several factors make China a critical region for this research. First, the widespread agricultural use of antibiotics, particularly in the animal farming sector, significantly contributes to the development and spread of antibiotic-resistant bacteria in the environment.[41–44] Second, the rapid urbanization and heavy industrialization seen in many Chinese regions result in a high volume of wastewater containing antibiotic-resistant bacteria, further exacerbating the problem. These factors, coupled with growing public health concerns regarding the increasing prevalence of resistant bacteria in both human populations and environmental ecosystems, underscore the importance of closely monitoring colistin resistance in China's wastewater systems. Therefore, our study aims to explore how these local challenges specifically influence the spread of resistance, with the goal of providing valuable data to guide targeted interventions.

This study aimed to investigate the prevalence, genomic characteristics, and horizontal gene transfer potential of *mcr*-carrying *Enterobacteriaceae* isolates from the influent of the Yangzhou Wastewater Treatment Plant in China, contributing to the understanding of their epidemiology and transmission dynamics within this environmental context.

Materials and Methods

Sample Collection and Bacterial Isolation

From March 2022 to January 2024, a total of 366 sewage samples were collected from the influent of the Yangzhou Wastewater Treatment Plant in Jiangsu Province, China. Briefly, 5 mL of each sewage sample was enriched in 25 mL of sterile Luria-Bertani broth and incubated overnight at 37°C with agitation at 180 rpm. Subsequently, 10 μL of the enriched sample was streaked onto Eosin-Methylene Blue Agar plates supplemented with 2 mg/L colistin. After 18 to 24 hours of incubation at 37°C, colonies with distinct morphologies were selected and further purified. Bacterial species were identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany).

PCR Detection of Mcr Genes

The presence of *mcr* genes (*mcr-1* to *mcr-10*) in colistin-resistant isolates was confirmed by PCR using specific primers, as previously described.[15,45,46]

Antimicrobial Susceptibility Testing

The minimal inhibitory concentrations (MIC) of 15 antibiotics were determined using agar dilution or broth microdilution methods (specifically for colistin and tigecycline) in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The tested antibiotics included ampicillin, cefotaxime, meropenem, gentamicin, amikacin,

streptomycin, tetracycline, tigecycline, nalidixic acid, ciprofloxacin, colistin, trimethoprim-sulfamethoxazole, chloramphenicol, florfenicol, and fosfomycin. The results were interpreted according to the 2022 CLSI guidelines (document M100-S32), except for the breakpoints of streptomycin, tigecycline, and florfenicol, which were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (https://www.eucast.org/). *E. coli* ATCC 25922 was used as the quality control strain.

Conjugation Experiments

Conjugation experiments were conducted using the *mcr*-positive isolates as donor strains, and *E. coli* C600 (streptomycin-resistant) as the recipient strain, following a previously described protocol.[47] Transconjugants were selected on LB agar plates supplemented with streptomycin (3000 mg/L) and colistin (2 mg/L). PCR was performed to confirm the presence of the *mcr* genes in the transconjugants.

Whole-Genome Sequencing and Analysis

Genomic DNA from *mcr*-positive isolates was extracted and sequenced using the Illumina NovaSeq and Oxford Nanopore MinION platforms. Hybrid *de novo* assembly was performed using SPAdes 3.11[48] and Unicycle 0.4.9,[49] with corrections applied using Pilon 1.23.[50] Genome annotations were conducted with Prokka 1.13.[51] Antimicrobial resistance (AMR) genes, plasmid replicons and mobile genetic elements were identified using tools from the Center for Genomic Epidemiology (https://cge.food.dtu.dk/). *In silico* multilocus sequence typing (MLST) was performed using the Institut Pasteur MLST online database (https://bigsdb.pasteur.fr/). Alignment of the *mcr*-bearing plasmids was visualized using the BLAST Ring Image Generator (BRIG) (http://brig.sourceforge.net/),[52] and the genetic context comparisons were visualized using the Easyfig tool (http://mjsull.github.io/Easyfig/).[53] Reference genomes were collected from the NCBI Genomic Database. The phylogenetic tree based on core genome single nucleotide polymorphisms (SNPs) was generated using ParSNP v. 1.2 (https://github.com/marbl/parsnp),[54] and the tree was visualized using ChiPlot (http://chiplot.online/#).[55]

Results

Isolates Identification and Resistant Phenotypes

A total of 33 colistin-resistant strains were isolated from the 366 sewage samples. Among these, three *mcr*-positive isolates were identified, corresponding to an isolation rate of 0.82%. These included one isolate harboring *mcr-1* (YZ22WS208) and two isolates harboring *mcr-10* (YZ22WS067 and YZ22WS223). Species identification was performed using MALDI-TOF MS, which confirmed YZ22WS208 as *E. coli*, while YZ22WS067 and YZ22WS223 were identified as *Klebsiella quasipneumoniae subsp. similipneumoniae* and *Klebsiella variicola subsp. variicola*, respectively.

Antimicrobial susceptibility testing revealed distinct resistance profiles between the *E. coli* and *Klebsiella* strains. The *Klebsiella* strains, YZ22WS067 and YZ22WS223, were resistant to and ampicillin, chloramphenicol and colistin but were susceptible to all other antibiotics tested, including cefotaxime, meropenem, gentamicin, amikacin, streptomycin, tetracycline, tigecycline, florfenicol, nalidixic acid, ciprofloxacin, fosfomycin, and trimethoprim-sulfamethoxazole. In contrast, *E. coli* strain YZ22WS208 exhibited a broader, more extensive resistance pattern, demonstrating resistance to a wide range of antibiotics, including ampicillin, cefotaxime, meropenem, gentamicin, amikacin, streptomycin, tetracycline, chloramphenicol, florfenicol, nalidixic acid, ciprofloxacin, colistin, and trimethoprim-sulfamethoxazole. Notably, YZ22WS208 was only susceptible to fosfomycin and tigecycline, which highlights the significant differences in antimicrobial resistance profiles between the *E. coli* and *Klebsiella* strains (Table 1).

Transferability of Mcr-1 and Mcr-10 Gene

Horizontal transfer of *mcr* gene was confirmed through conjugation experiments. The *E. coli* strain YZ22WS208 successfully transferred *mcr-1* to the recipient strains, resulting in transconjugants that retained colistin resistance, with a MIC value of 8 mg/L (Table 1). The transfer frequency of *mcr-1* was determined to be 2.0462×10^{-2}. In contrast, the *mcr-10* genes present in the *Klebsiella* strains did not demonstrate detectable transferability in this study.

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Table 1 Antimicrobial Susceptibility Patterns in YZ22WS067, YZ22WS223, YZ22WS208 and Its Transconjugant

Strain	Species	MIC (mg/L)[a]														
		AMP	CTX	MEM	GEN	AMK	STR	TET	TIL	CHL	FFC	NAL	CIP	CL	FOS	SXT
YZ22WS067	*K. quasipneumoniae subsp. similipneumoniae*	>128	0.5	0.06	1	2	2	1	0.5	32	2	16	0.125	4	16	1
YZ22WS223	*K. variicola subsp. variicola*	128	0.5	0.03	1	1	1	2	0.5	32	2	16	0.125	4	64	1
YZ22WS208	*E. coli*	>128	>128	32	>128	>256	128	>128	0.5	>128	128	>256	>64	8	1	32
JYZ22WS208	*E. coli*	>128	>128	0.03	>128	>256	>256	4	0.25	4	4	4	0.25	8	1	32
C600	*E. coli*	8	0.06	0.015	0.25	0.5	>256	1	0.125	2	2	2	0.008	0.25	1	1

Abbreviations: AMP, ampicillin; CTX, cefotaxime; MEM, meropenem; GEN, gentamicin; AMK, amikacin; STR, streptomycin; TET, tetracycline; TIL, tigecycline; CHL, chloram-phenicol; FFC, florfenicol; NAL, nalidixic acid; CIP, ciprofloxacin; CL, colistin; FOS, fosfomycin; SXT, trimethoprim-sulfamethoxazole.

Genomic Features of Mcr-1-Positive Isolates

Multilocus sequence typing (MLST) identified *E. coli* YZ22WS208 as sequence type ST167 (Table 2), which is commonly associated with the dissemination of resistance genes. Whole-genome sequencing revealed that YZ22WS208 comprised a 4,853,249-bp chromosome and five plasmids. The chromosome contained six antimicrobial resistance genes, including *aph(4)-Ia, aadA1, aadA2b, aac(3)-IV, cmlA1* and *sul3*. Plasmid pYZ22WS208_1, harboring the carbapenem resistance gene $bla_{\text{NDM-5}}$, was classified as an IncHI2 plasmid. The plasmid showed high similarity to other NDM-producing IncHI2 plasmids found in *Enterobacteriaceae*, including *E. coli, K. pneumoniae*, and *Salmonella enterica* subsp. *enterica serovar* Typhimurium, from both animal and human origins (Figure 1A). Plasmid pYZ22WS208_2 was categorized as an IncFIB/IncFIC hybrid plasmid and carried five drug resistance genes. Sequence analysis demonstrated 99.75% identity and 87–89% coverage with six previously reported IncFIB/IncFIC plasmids from *E. coli* strains (eg, pEC-10, pAR349, pCUVET20-1667.1, pYLPK12, pMCR1-PA and pMR0516mcr). These plasmids shared a common backbone region that included several resistance genes, such as $bla_{\text{CTX-M-55}}$, as well as replication initiation gene *repB* and other essential genetic elements (Figure 1B). Plasmid pYZ22WS208_3 was identified as a multireplicon plasmid with an IncI2 replicon. It showed high similar to pHNSHP45, the first plasmid reported to carry *mcr-1* (Figure 1C). The genetic environment surrounding the *mcr-1*-flanking region in pYZ22WS208_3 showed 99.98% identity to that of pHNSHP45 (accession number KP347127.1). However, the transposable elements IS*Apl1* and IS*683* found upstream of *mcr-1* in pHNSHP45, were absent in pYZ22WS208_3. Additionally, pYZ22WS208_3 carried the $bla_{\text{CTX–M–99}}$ gene, which was located within a typical transposition unit (IS*Ecp1*–$bla_{\text{CTX–M–199}}$–orf477) (Figure 1D).

Genomic Features of Mcr-10-Positive Isolates

MLST analysis identified that two strains of *Klebsiella pneumoniae* complex, YZ22WS067 and YZ22WS223, belonged to novel sequence types ST6801 and ST6825, respectively (Table 2). The genome of YZ22WS067 consisted of a 5,162,045-bp chromosome and six plasmids, while YZ22WS223 harbors a 5,575,338-bp chromosome and two plasmids

Table 2 Genomic Characteristics of Mcr-Carrying Enterobacteriaceae Isolates

	Size (bp)	GC Content (%)	ST	Resistance Genes	Plasmid Replicon
YZ22WS067					
Chromosome	5,162,045	57.78	6801	*fosA, oqxa, oqxb,* $bla_{\text{OKP-B-1}}$	–
pYZ22WS067_1	250,618	52.05		*mcr-10*	IncFIB-IncFII
pYZ22WS067_2	151,498	52.24		–[a]	IncFII-$\text{IncF}_{\text{repB(R1701)}}$
pYZ22WS067_3	5,782	48.04		–	–
pYZ22WS067_4	4,048	54.09		–	Col440II
pYZ22WS067_5	1,702	40.92		–	–
pYZ22WS067_6	1,240	45.92		–	–
YZ22WS223					
Chromosome	5,575,338	57.22	6825	*fosA, oqxa, oqxb,* bla_{LEN16}	–
pYZ22WS223_1	192,277	52.34		*mcr-10*	$\text{IncF}_{\text{repB(R1701)}}$
pYZ22WS223_2	11,727	51.03		–	–
YZ22WS208					
Chromosome	4,853,249	50.70	167	*aph(4)-Ia, aadA1, aadA2b, aac(3)-IV, cmlA1, sul3*	–
pYZ22WS208_1	270,188	47.26		*aph(4)-Ia, aph(6)-Id, aph(3')-Ia, aph(3")-Ib, aadA22, aadA1, aac(3)-IV,* $bla_{\text{TEM-1B}}$, $bla_{\text{NDM-5}}$, $bla_{\text{OXA-10}}$, *lnu(F), cmlA1, floR, qnrS1, arr-2, tet*(A), *dfrA14*	IncHI2
pYZ22WS208_2	123,987	49.81		*aph(3')-IIa, rmtB,* $bla_{\text{CTX-M-55}}$, $bla_{\text{TEM-1B}}$, *dfrA14*	IncFIB-IncFIC
pYZ22WS208_3	68,405	42.73		$bla_{\text{CTX-M-199}}$, *mcr-1.1*	IncI2
pYZ22WS208_4	3,373	55.25		–	–
pYZ22WS208_5	3,138	51.03		–	–

Notes: [a], no resistance genes or plasmid replicon were found.

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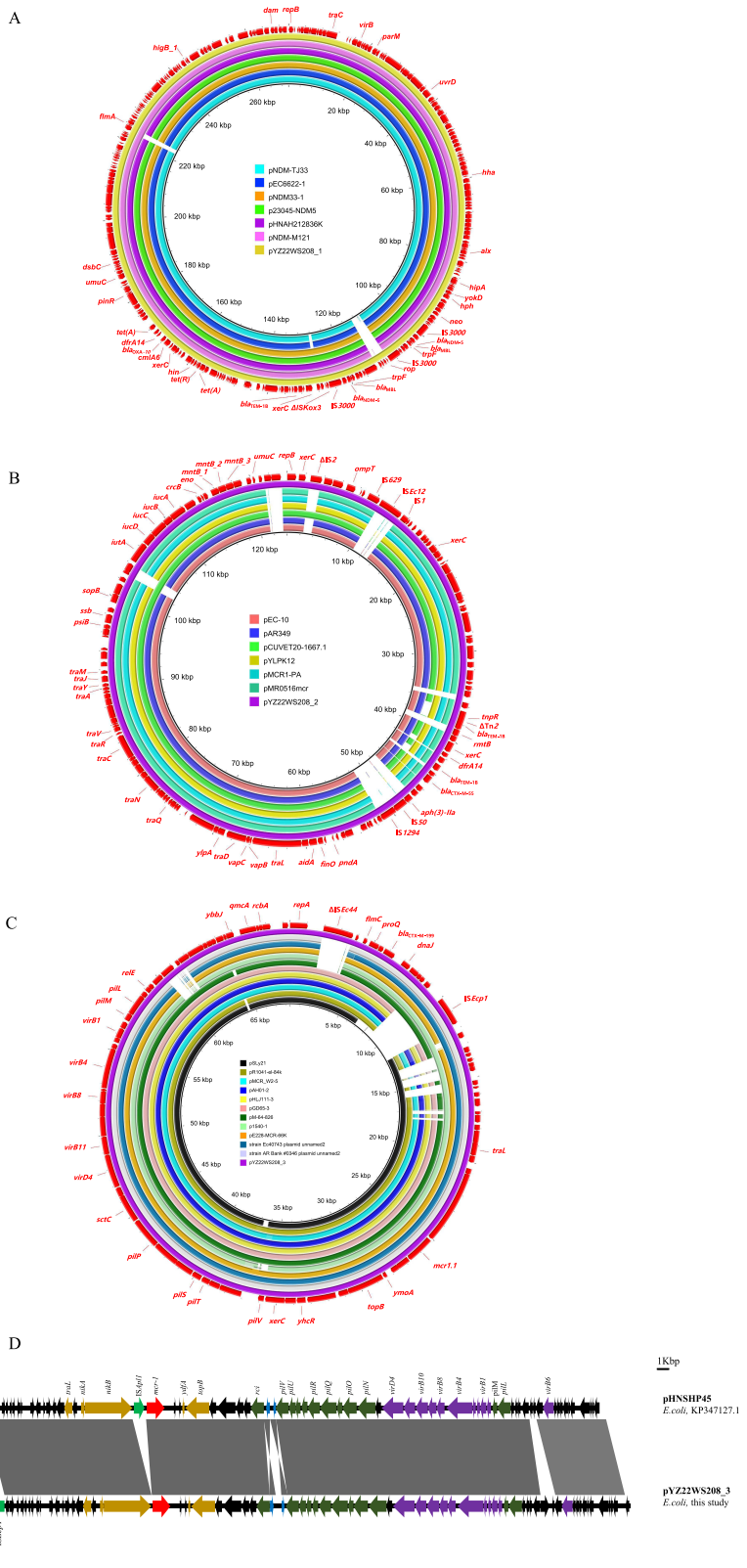

Figure 1 Comparison of YZ22WS208 plasmids with those available in the NCBI database. (**A**) Comparison of pYZ22WS208_1 in this study with other plasmids pNDM-TJ33 (MN915010.1), pEC6622-1(CP096588.1), pNDM33-1(MN915011.1), p23045-NDM5(OR497833.1), pHNAH212836K(CP104628.1), pNDM-M121(CP083586.1). (**B**) Comparison of pYZ22WS208_2 in this study with other plasmids pEC-10(CP065204.1), pAR349(CP041997.1), pCUVET20-1667.1(CP115362.1), pYLPK12(CP074032.1), pMCR1-PA(CP029748.1), pMR0516mcr (KX276657.1). (**C**) Comparison of pYZ22WS208_3 in this study with other plasmids pSLy21(CP016405.1), pR1041-el-84k(OR095748.1), pMCR_W2-5(CP032990.1), pAH01-2(CP055253.1), pHLJ111-3(MN232207.1), pGD65-3(KY075661.1), pM-64-826(MT773675.1), p1540-1(CP019052.1), pE228-MCR-66K(CP150077.1), strain Ec40743 plasmid unnamed2(CP041921.1), strain AR Bank #0346 plasmid unnamed2(CP066368.1). (**D**) Schematic representation of the genetic environments flanking the *mcr-1*-flanking region in pYZ22WS208_3 and pHNSHP45 (KP347127.1). Arrows indicate transcription direction, with different gene colors, and regions with ≥ 90.0% nucleotide identity shaded gray.

(Table 2). Both isolates carried the same antimicrobial resistance genes on their chromosomes, including *fosA* and *oqxAB*. However, chromosomal beta-lactam resistance genes differed between the two strains, with YZ22WS067 carrying $bla_{\text{SHV-182}}$ and YZ22WS223 harboring $bla_{\text{LEN-16}}$. Both strains carried the *mcr-10* gene on plasmids pYZ22WS067_1 (250,618 bp) and pYZ22WS223_1 (192,277 bp), respectively, with no additional resistance genes identified on the other plasmids.

A BLASTN comparison between the *mcr-10* plasmids identified in this study and those available in the GenBank database revealed that pYZ22WS067_1 shared over 61% coverage and more than 99.5% identity with four *mcr-10*-carrying plasmids: three from *K. pneumoniae* (pMyNCGM088, pMyNCGM084, pKP46-mcr10, pKP57-mcr10), and one from *Raoultella ornithinolytica* (strain FDAARGOS_431, plasmid unnamed1). Similarity, pYZ22WS223_1 exhibited over 57% coverage and 100% identity three *mcr-10*-carrying plasmids: two from *K. pneumoniae* (pMyNCGM088, pMyNCGM084), and one from *R. ornithinolytica* (strain FDAARGOS_431, plasmid unnamed1) (Figure 2A). Despite the relatively low coverage, both plasmids contained an undiversified replicon, likely belonging to the IncF group (Table S1).

Comparative analysis of the two *mcr-10* positive plasmids in this study and those previously reported plasmids revealed structural differences in their surrounding genetic environment. In *E. coli*, in *K. pneumoniae* and *Enterobacter roggenkampii*, the basic plasmid backbone was conserved, with the *xerC* gene, encoding a tyrosine recombinase, located upstream of all *mcr-10* genes. Various insertion sequences were also identified upstream and/or downstream of the *mcr-10* genes (Figure 2B). IS*Ecp36* and a truncated IS*EcI1* were present downstream of *mcr-10* in pYZ22WS067_1 (Figure 2B), differing from the first reported *mcr-10*-positive plasmid, pMCR10_090065 (accession number CP045065), which contained two truncated IS*903B* sequences flanking *mcr-10*, forming a composite transposon. In contrast, pYZ22WS223_1 carried a more diverse set of insertion sequences downstream of *mcr-10*, including IS*Kox1*, IS*Ec27*, IS*Kpn42*, IS*sm1*, and a truncated IS*EcI*1.

Phylogenetic Analysis of Mcr-1-Bearing Isolates

A phylogenetic comparison was conducted on the *mcr-1*-positive *E. coli* isolate in this study, along with 38 published *E. coli* genomes from 17 countries, sourced from the NCBI database (Figure 3). The analysis revealed that all isolates clustered into two major evolutionary branches, exhibiting a wide range of sequence types.

Clade I comprised fourteen strains from nine countries, including Egypt, Pakistan, Italy, China, Lebanon, India, Singapore, the United Kingdom and Malaysia. These strains were isolated from a variety of sources, including humans, animals, and environmental samples, reflecting their widespread distribution across diverse ecological niches.

The *E. coli* isolate from this study clustered within clade II, alongside strains from India, Japan, China, Thailand, Bangladesh, Pakistan, Belgium, Brunei, Brazil, Italy, Austria and South Korea. In Clade II, the majority of *mcr-1*-harboring *E. coli* isolates was originated from environmental or animal sources. Notably, strain YZ22WS208 exhibited the closest phylogenetic relationship to a strain from human feces in Hangzhou, China. Both strains shared the same sequence type (ST167), indicating a potential link in their evolutionary history and suggesting a common source and transmission pathway.

Phylogenetic Analysis of Mcr-10-Bearing Isolates

To explore the prevalence and distribution of *mcr-10* in the *K. pneumoniae* complex, a phylogenomic analysis was performed based on core genomes SNPs. The phylogenetic tree included 43 *mcr-10*-positive *K. pneumoniae* complex isolates, comprising two strains from this study and 41 strains from the NCBI GenBank database (Figure 4). The analysis revealed that *mcr-10* is sporadically distributed across 13 countries, with a predominance of strains of human origin and a wide range of sequence types. The isolates were grouped into four distinct clades: Clade I (n=2, 4.65%), Clade II (n=12, 27.90%), Clade III (n=10, 23.26%), and Clade IV (n=19, 44.18%).

Strain YZ22WS067 clustered within clade I, along with strain 23-M-SRM-61, which was isolated from hospital wastewater in Zhejiang, China. Both strains were identified as *K. variicola subsp. variicola* (Kp3). Strain YZ22WS223 was closely related to strain SB610, which was isolated from water in New Zealand and clustered into Clade III. The remaining clades consisted of isolates from human, environmental, and animal sources. Clade II and III, which exhibited the greatest diversity, included *K. pneumoniae* (Kp1) and *K. quasipneumoniae subsp. similipneumoniae* (Kp4). Clade IV,

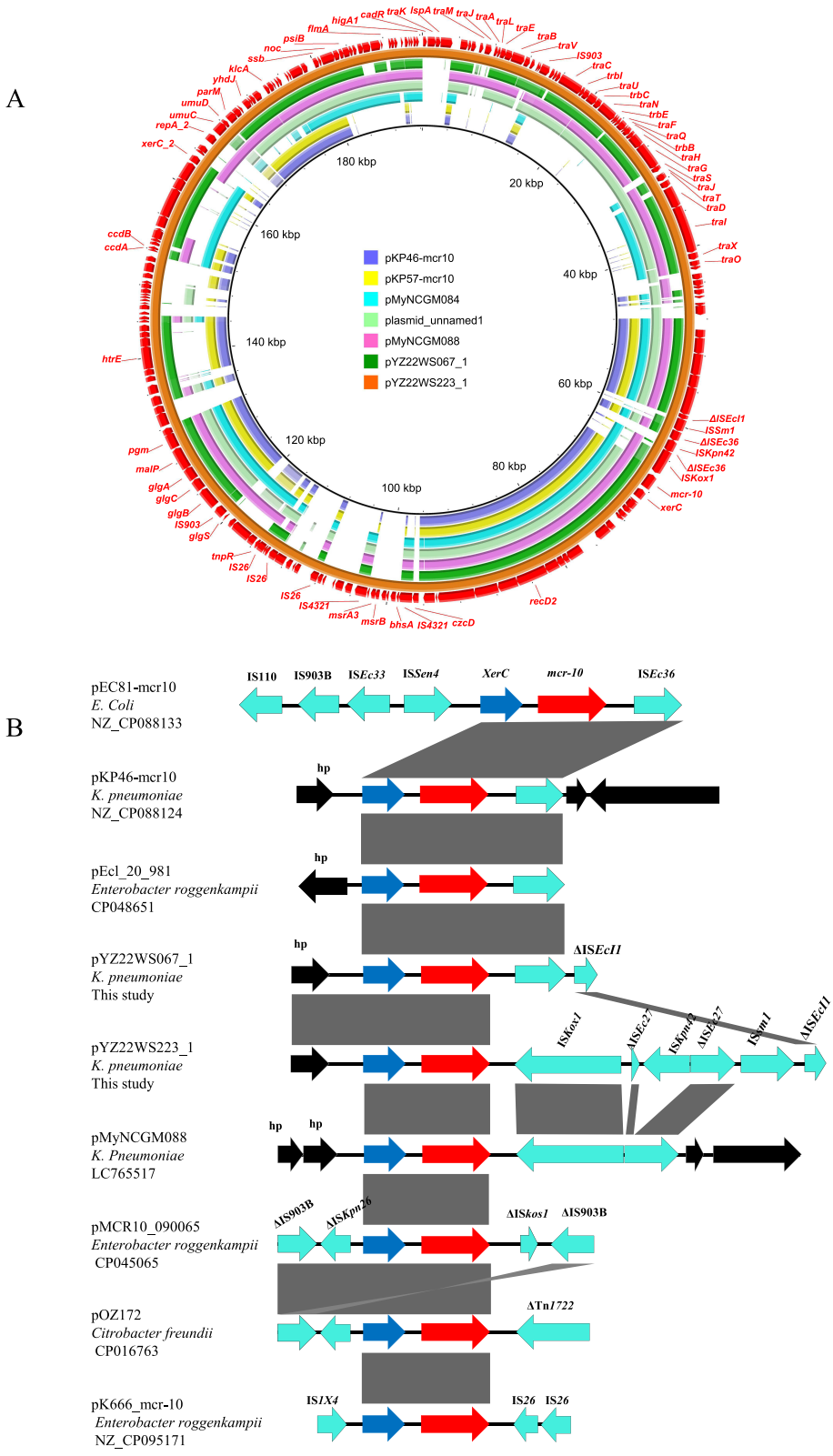

Figure 2 Sequence comparison of *mcr-10*-carrying plasmids and their surrounding genetic structures. (**A**) Circular alignment of seven complete *mcr-10* plasmids. Circles from the inside to outside indicate plasmids pKP46-mcr10 (CP088124.1), pKP57-mcr10 (CP088129.1), pMyNCGM084 (LC765516.1), plasmid_unnamed1(CP023893.1), pMyNCGM088 (LC765517.1), pYZ22WS067_1 (this study), and pYZ22WS223_1 (this study). (**B**) Comparison of two *mcr-10*-positive plasmids in this study with those in the NCBI database. Arrows indicate transcription direction, and different gene colors, and regions with ≥90.0% nucleotide identity shaded gray. Δ denotes a truncated gene.

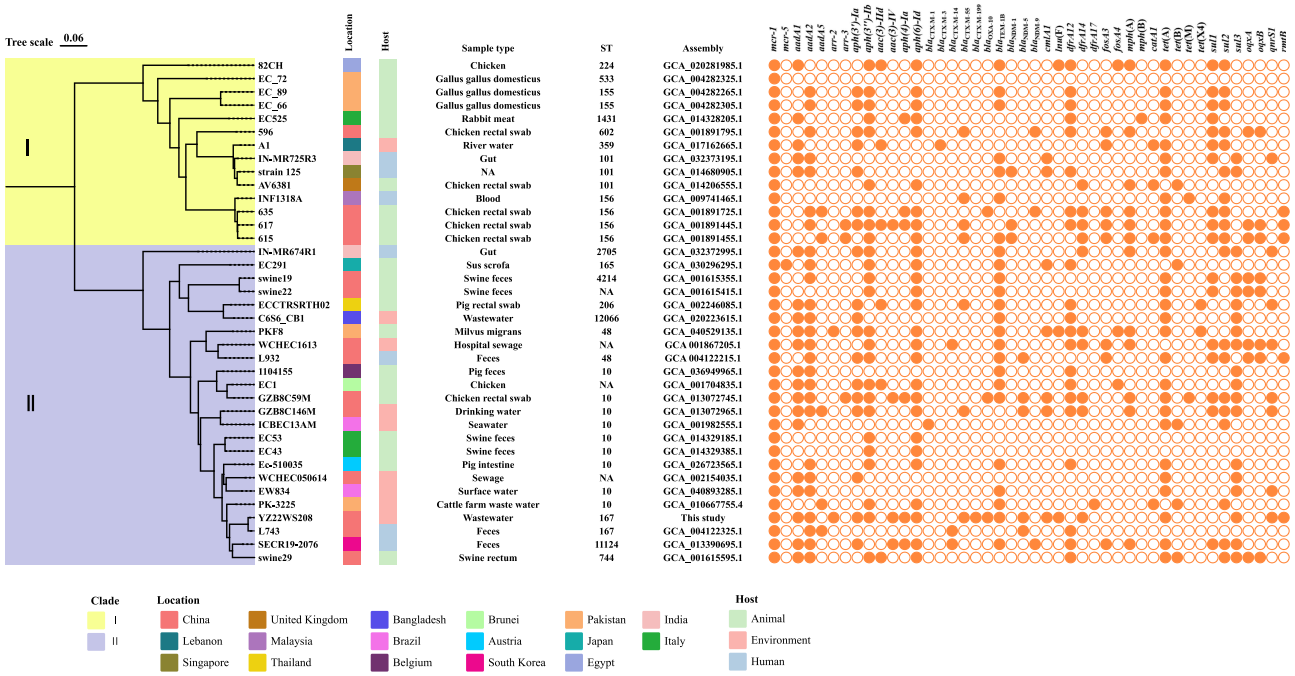

Figure 3 Phylogenetic relationships among 38 *mcr-1*-positive *E. coli* strains, with sequence type, source, and resistance genes indicated on the right.

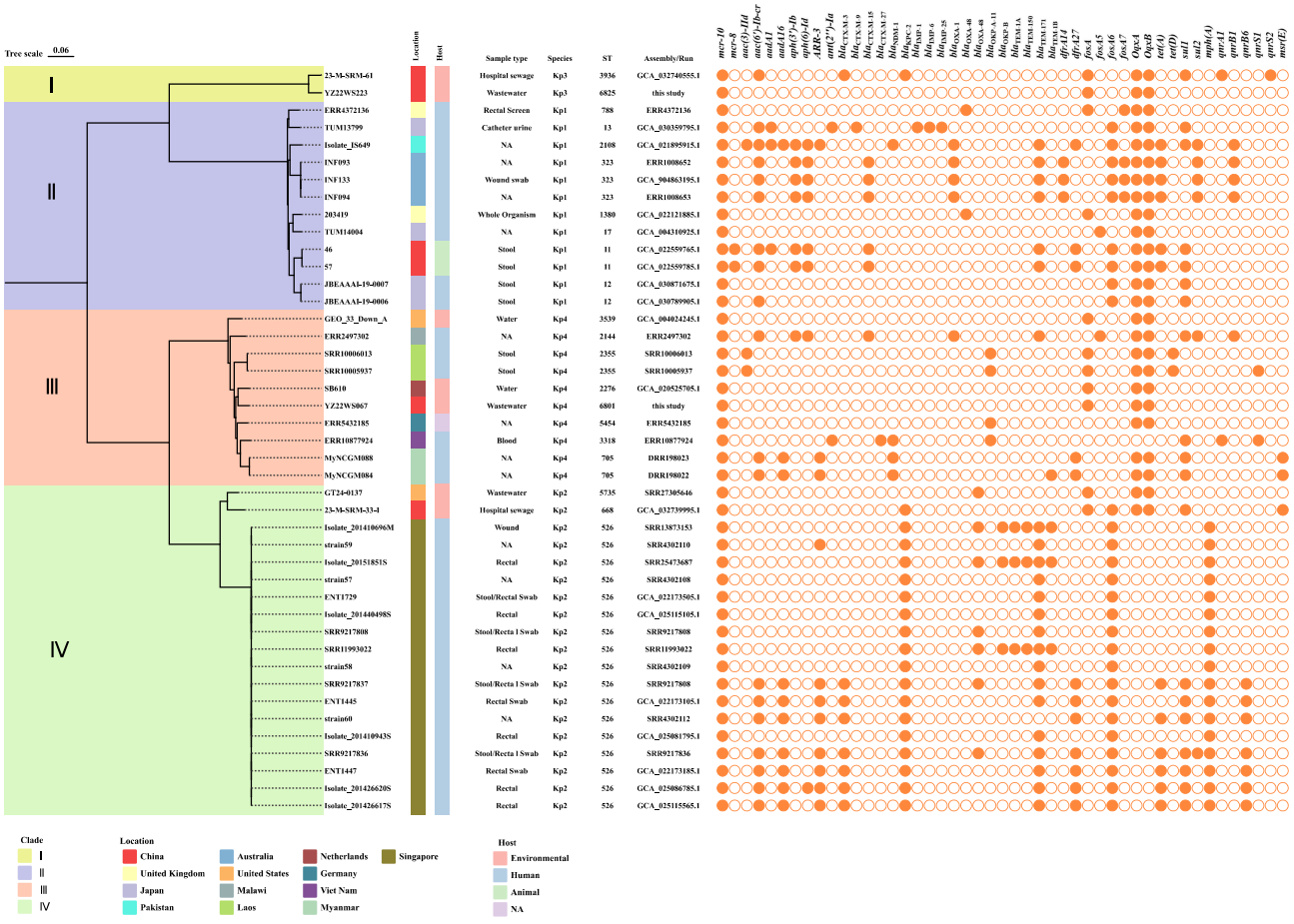

Figure 4 Phylogenetic relationships among 43 *mcr-10*-positive *Klebsiella pneumoniae* complex strains, with sequence type, source, and resistance genes indicated on the right.

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composed of *K. quasipneumoniae subsp. quasipneumoniae* (Kp2), contained strains from the USA, China, and Singapore, with the majority originating from Singapore, all sharing the sequence type ST526.

Discussion

Enterobacteriaceae strains carrying the *mcr* gene were isolated from 366 wastewater samples collected at the influent of a wastewater treatment plant in Yangzhou City, with an isolation rate of 0.82%. This study identified one *E. coli* strain harboring the *mcr-1* gene and two *K. pneumoniae* complex strains carrying the *mcr-10* gene. While the *mcr-10* gene has previously been reported in human samples, such as patients and slaughterhouse workers, as well as environmental samples, including hospital sewage, disinfected tableware, and animals like companion animals and chickens in China,[15,56–60] this is the first time that *mcr-10* has been detected in a wastewater treatment plant.

The *E. coli* strain YZ22WS208, which harbored the *mcr-1* gene, exhibited resistance to colistin, consistent with previous studies.[59,61] The two *K. pneumoniae* complex isolates carrying the *mcr-10* gene, YZ22WS067 and YZ22WS223, also showed resistance to colistin. Some studies report that *mcr-10*-positive strains do not show phenotypic colistin resistance,[15,57] while others have observed high levels of resistance.[58,59,61] This discrepancy suggests that the expression of the *mcr-10* gene may be closely linked to the genetic background of individual strains. The complexity of antibiotic resistance mechanisms underscores the need for further investigation into the relationship between the *mcr* genes and colistin resistance. The emergence of *K. pneumoniae* complex carrying the *mcr-10* gene in Yangzhou highlights a concerning development, emphasizing the need to remain vigilant about the potential threat posed by the *mcr* gene. The spread and evolution of the *mcr* gene is a dynamic process, and highly resistant strains may emerge in the future, posing a serious threat to public health.

Previous studies have shown that plasmids carrying the *mcr-10* gene are primarily found in *Enterobacter spp*.,[15,58,62,63] suggesting that *mcr-10*-harboring isolates may exhibit stable genus specificity. The two *K. pneumoniae* complex strains YZ22WS067 and YZ22WS223, isolated from sewage, present novel sequence types. Data from domestic and international reports of *mcr-1*-carrying isolates from sewage align with our findings, with *E. coli* being the main carrier of the *mcr-1*-gene.[64–68] *E. coli* isolates with various sequence types (STs) carrying *mcr-1* have been identified in animals, foods, humans and environmental samples.[10,69–73] The ST167 identified in this study has also been reported as a common *mcr-1* carrier in humans and sewage in China.[40,74–76] The *E. coli* YZ22WS208, isolated from Yangzhou sewage, harbored not only the *mcr-1* gene and $bla_{\text{CTX-M-55}}$ but also $bla_{\text{NDM-5}}$ and $bla_{\text{OXA-10}}$. YZ22WS208 exhibited carbapenem resistance, which was confirmed to be due to the production of the carbapenemase NDM-5, as identified by whole-genome sequencing. This gene is located on the IncHI2 plasmid pYZ22WS208_1 and is consistent with other studies reporting NDM-5-mediated resistance in *Enterobacteriaceae*. However, for the two *Klebsiella* isolates (YZ22WS067 and YZ22WS223), antimicrobial susceptibility testing indicated that they were not resistant to carbapenems. This finding aligns with the absence of carbapenemase genes in these isolates. Outside of China, *E. coli* ST167 *mcr-1*-positive has also been reported. For example, in 2016, an *E. coli* ST167 strain carrying *mcr-1* was isolated from the sputum of a pneumonia patient in Spain.[77] In 2019, Yoko Nukui et al reported the first isolation of an *E. coli* ST167 strain co-harboring $bla_{\text{NDM-5}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{OXA-10}}$, and *mcr-1* from the sputum of a Japanese pneumonia patient.[78] To date, *E. coli* ST167 has been classified as an internationally disseminated clonal lineage associated with the global spread of CTX-M broad-spectrum β-lactamases and NDM metallo-β-lactamases in humans and animals.[79]

Plasmids play a critical role in the global spread of resistance genes, including *mcr-1* in *Enterobacteriaceae*.[10] Three plasmid incompatibility groups, including IncI2, IncX4 and IncHI2, have been identified as vectors of *mcr-1*.[10,80] Consistent with previous findings, the presence of *mcr-1* in the IncI2 plasmid was detected in *E. coli* YZ22WS208, which shared 99.98% identity with plasmid pHNSHP45 (accession number KP347127.1). The plasmid pYZ22WS208_3 identified in this study possesses conjugative capabilities, increasing the risk of *mcr-1* transmission between bacteria. Additionally, the bacterium carries IncF and IncHI2 plasmids that harbor other resistance genes. Although the conjugation experiment was unsuccessful, there remains a possibility that these resistance genes could transfer in the future through mediation by other insertion elements. This is concerning, as such events could lead to infections that are difficult to treat and pose significant public risks.

In 2022, Yin et al summarized the genetic background associated with *mcr-10*. This study confirmed that in YZ22WS067 and YZ22WS223, *mcr-10* gene was located in a similarity conserved structure, with *xerC* located upstream and IS*Ec36*/IS*26* positioned downstream of the core *xerC-mcr-10* structure in most strains.[59] Consistent with these findings, the genetic environment of *mcr-10* in strains YZ22WS067 and YZ22WS223 was consistent with this conserved arrangement.

Furthermore, phylogenetic analyses revealed a close relationship between *mcr-10* and *mcr-1*-carrying isolates from human, animal, and the environmental sources. This suggests that *mcr*-positive plasmid isolates may have the potential to spread across different hosts and environmental boundaries. To better understand this threat, further research is crucial to identify the transmission routes of *mcr-10*-positive and *mcr-1*-positive *Enterobacteriaceae* cross these ecosystems. Additionally, more epidemiological studies are needed to elucidate the transmission patterns of *mcr*-positive *Enterobacteriaceae* in diverse populations. By investigating these pathways, we can implement more effective measures to control the spread of *mcr* genes and mitigate the public health risks posed by antibiotic resistance.

Conclusion

This study detects *mcr-1*-positive *E. coli* and *mcr-10*-positive *K. pneumoniae* complex strains in wastewater in Yangzhou, China. The presence of *mcr*-positive *Enterobacteriaceae* isolates in wastewater highlights the potential risk of gene spread, even in environments with limited colistin exposure. Early detection and ongoing monitoring are crucial, and wastewater surveillance is a valuable tool for tracking antibiotic resistance across ecosystems.

Data Sharing Statement

The genome sequences in this study have been deposited into NCBI GenBank under PRJNA1162414.

Ethical Statement

This study does not involve any direct interaction with animals or human subjects, nor does it collect private or identifiable information. In accordance with the "Measures for the Ethical Review of Science and Technology" (Trial), issued jointly by the Ministry of Science and Technology, Ministry of Education, and other departments of the People's Republic of China under document number Guo Ke Fa Jian [2023] No. 167, this research does not require ethical approval as it does not involve human or animal subjects nor sensitive data. Additionally, we confirm that permission for the collection of wastewater samples was granted by Mr. Qingbo Shang, Director of the Tangwang Wastewater Treatment Plant, Yangzhou Jieyuan Environmental Co., Ltd. Mr. Shang approved the sampling work and arranged for staff to assist with the wastewater collection.

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Disclosure

The authors report no conflicts of interest in this work.

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